Isolation and characterization of bioactive compounds with special reference to anti inflammatory potential of *Centella asiatica*

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Abstract— Living tissues, when injured or infected will produce inflammatory responses. Lysosomal enzymes released during inflammation causes plenty of disorders, which leads to tissue injury by damaging the macromolecules and lipid peroxidation of membranes. These are assumed to be responsible for certain pathological conditions as heart attacks, septic shocks and rheumatoid arthritis etc. The extracellular activities of these enzymes are said to be related to acute or chronic inflammation. It has been reported that the presence of protease inhibitors at the site of inflammation has a crucial role in limiting the destructive activity of proteases that are liberated from polymorphonuclear leukocytes and other necrotizing tissues. The inhibition of these proteases by other normally occurring inhibitors and synthetic ones could be one of the significant steps in preventing tissue damage during inflammation. This study supports the isolation and use of active components from *Centella asiatica* in treating inflammatory responses. *C. asiatica* is a medicinal herbaceous aromatic creeper which has been valued for centuries in ayurveda. Phytochemical analysis of *C. asiatica* leaf extracts revealed the presence of different biochemical compounds such as alkaloids, flavonoids, glycosides, triterpenoids and saponins etc. The Bioassay-guided column chromatography of the plant extract was done using hexane-chloroform as mobile phase and the active fractions were examined by TLC using toluene: ethyl acetate (2:8) as mobile phase. The GC-MS of the active fraction identified the active compounds in the extract was Phytol. The proteinase inhibitory assay of the purified compound shows 67.29% inhibition were as the standard inhibitor (PMSF) shows an inhibition of 53.2%. Molecular docking studies revealed that the compounds are thermodynamically feasible and has significant glide scores. The ADMET analysis shows that the compound obeys the Rule of Five with one violation (MLOGP>4.15)..

Index Terms— Anti-inflammatory, *Centella asiatica*, Molecular docking, GC-MS.

1 INTRODUCTION

Herbs are major component of traditional, ayurvedic, Unani, homeopathic and naturopathic medicines [1]. There is a belief that natural remedies are superior to man-made drugs because they are always associated with natural and biological entities like protein, lipids and carbohydrates [2]. The World Health Organization (WHO) estimated that at least 80% of the world's population depends on traditional system of medicine for their primary health needs. These systems are largely plant based due to the growing awareness about side effects and complications of chemical and synthetic medicines [3].

The plant is generally known as Asiatic pennywort, Indian pennywort or Spade leaf and belongs to Umbelliferae/Apiaceae family. In China, Southeast Asia, India, Sri Lanka, Oceania, and Africa, this plant has been used as vegetable. In Southeast Asia, it is traditionally used for curing a wide variety of disorders such as skin diseases, rheumatism, inflammation, syphilis, mental illness, epilepsy, hysteria, dehydration, and diarrhea [4]. *Centella asiatica* is used in Indian medicinal system for enhancing memory and for the treatment of skin diseases and nervous illness. The plants medicinal properties have long been understood and utilized by the people of Java and Indonesia. In kerala, it is traditionally called as Kudangal, and over 2000 years ago [5]. Herbal medicines can be utilized as adaptogens, the plant derived drugs either reduce stress reactions in the alarm phase and provide a certain degree of safety against long-term stress [6]. *C. asiatica* (Umbelliferae) syn. Hydrocotyle asiatica is used to cure various ailments across India, which includes body aches, headaches, insanity, asthma, leprosy, ulcers, eczemas, and wound healing [7]. For new potential compounds for therapeutic purpose, screening of medicinal plant is vital [8].

This work was part of the scientific validation of the ethnopharmacological claim about the anti-inflammatory properties of leaf extract. An attempt to evaluate the *in vitro* anti-inflammatory activities of methanolic extracts of *C. asiatica* was made using proteinase inhibition studies. The effects were compared to phenyl methane sulfonyl fluoride (PMSF) as standard and the active fraction was isolated through column chromatography with hexane-chloroform as mobile phase. The compound was identified using the GC-MS and molecular docking studies were done using the crystal structure of trypsin using Maestro 9.4 suite of Schrodinger program

2 MATERIALS AND METHODS

2.1 Plant materials collection and identification

The *C. asiatica* leaves was collected from Kannur University campus, Palayad, Kerala (11°47'46.9"N 75°28'03.7"E) and herbarium was prepared. The shade dried; powdered plants

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were stored in airtight container.

2.2 Extraction of plant material

The powdered plant material (60 g) was extracted with methanol (60℃-80℃), using Soxhlet apparatus for 72 h. The extracts were concentrated under reduced pressure using rotary vacuum evaporator and stored at 2℃- 8℃ until the completion of pharmacological studies. yield of CAMLE alone was 21.62% w/w.

2.3 Phytochemical Screening

The extracts were then subjected to preliminary phytochemical screening using standard procedures [9]. The phytochemical screening was carried out using phytochemical tests or reagents like Mayer's reagent, Hager's reagent (saturated picric acid sol.), Wagner's reagent and Dragendorff's reagent for alkaloids; Molisch's test, Benedict's test and Fehling's test for carbohydrates; Legal's test and Baljet test for cardiac glycosides; foam test for saponins glycosides; Salkowski's test for steroids; dilute FeCl3 sol. (5%) test and lead acetate test for phenolic compounds and tannins; Millon's test, Biuret test and ninhydrin test for proteins and amino acids; oil stain test for fixed oil; Salkowski test and Liebermann-Burchard test for sterols and steroids; lead acetate test and Mg and HCl tests for flavonoids.

2.4 Protease inhibitory activity

The serine protease trypsin was used for the assay of plant extract. The trypsin inhibition assay was carried out using the spectrophotometric assay developed by Sigma Aldrich with slight modifications. In this procedure the activity was determined by Nα-Benzoyl-L-arginine ethyl ester (BAEE) as the substrate. The procedure is a continuous spectrophotometric rate determination (A253, Light path = 1 cm). The reaction mixture contains 67 mM phosphate buffer (pH 7.6), 0.25 mM BAEE in phosphate buffer and 0.05 mM trypsin in 1 mM ice cold HCL. The extracts were prepared in DMSO and used for the assay. The assay mixture that contains 200µl of trypsin and 200µl of test solution was incubated for 10 minutes. The reaction was initiated by the addition of 3 ml of the substrate and the absorbance was measured at 253 nm for 10 minutes using UV visible spectrophotometer. PMSF is used as positive control. The percentage of inhibition was calculated by the following equation.

Percentage inhibition = (Abs control –Abs sample) X 100/Abs control

2.5 Chromatographic techniques

The methanol fraction showing protease inhibitory activity was subjected to column chromatography using silica gel (mesh 230-400). Initially the column was eluted using hexane (50ml), followed by hexane: chloroform (48:2) etc up to 50 ml chloroform by gradient elution technique. Each fraction was analysed for protease inhibitory activity. From the 25 fractions collected, one fraction was found positive for protease inhibition.

2.6 PTLC

The active fraction obtained from column chromatography were subjected to preparative thin layer chromatography (PTLC). Separation was carried out on glass TLC plates coated with silica gel-G with Toluene: Ethyl acetate (2:8) as mobile phase. The bands were then scraped off, dissolved in chloroform and filtered. The solvents were removed by evaporation and the samples were tested for the protease inhibition. The samples were further subjected for GC-MS analysis for the identification of the compounds

2.7 GC-MS analysis

The Trace GC Ultra and DSQII model MS from Thermo Fisher Scientific Limited, were engaged for analysis. The instrument was set as follows, Injector port temperature set to 250℃, Interface temperature set as 250℃, source kept at 220oC. The oven temperature programmed as available, 70oC for 2 mins, 150 oC @ 8oC/min, up to 260oC @ 10oC/min. Split ratio set as1:50 and the injector used was splitless mode. The DB-35 MS Nonpolar Column was used whose dimensions were 0.25 mm OD x 0.25 µm ID x 30 meters length procured from Agilent Co. USA. Helium was used as the carrier gas at 1 ml/min. The MS was set to scan from 50 to 650 Da. The source was maintained at 200oC and <40 mtor vacuum pressure. The ionization energy was -70eV. The ME was also having inbuilt pre-filter which reduced the neutral particles. The data system has two inbuilt libraries for searching and matching the spectrum. NIST4 and WILEY9 each contain more than five million references. Only those compounds with spectral fit values equal to or greater than 700 were considered positive identification.

Interpretation of mass spectrum of GC – MS was done using the database of National Institute Standard and Technology (NIST4) and WILEY 9. The spectrum of the known component was compared with the spectrum of the known components stored in the inbuilt library.

2.8 Molecular docking studies of the selected compounds with trypsin

Molecular docking studies were carried out with the structure interpreted by GC-MS analysis. Maestro 9.4 suite of Schrodinger program was used for in silico flexible docking studies (Schrodinger Inc., USA). The crystal structure of the bovine trypsin was obtained from the Protein Data Bank, submitted under the ID 3LJJ. The structures of the ligands namely Phenylmethylsulfonyl fluoride (PMSF) and phytol were obtained from pubchem database.

The crystal structure of the protein was prepared for docking using the protein preparation wizard of schrodinger program. Water molecules from the structure were deleted and polar hydrogens were added to the protein. The structure was then minimized using OPLS 2005 force field. The minimisation process was terminated when the root mean square deviation of the minimised structure relative to the crystal structure exceeded 0.3Å. The structures of various ligands were prepared and optimized for docking studies by Ligprep module of Schrodinger program with MMFF force field.

A 20 Å grid was set based on the position of the ligand quercetin in the crystallographic structure of the PDB (3LJJ). The amino acid residues val 213, ser214, trp215, gly216, gly219, cys220, leu99, thr96, asn97 etc. were found in the grid. XP docking was conducted using the appropriate options in the software.

2.9 ADMET parameters prediction

FADMET parameters were predicted using pkCSM web server [10]. ADME parameters such as water solubility, CaCo-2 permeability, intestinal absorption, P-glycoprotein, the volume of distribution, blood-brain barrier (BBB) and CNS permeability along with Toxicity parameters such as AMES toxicity (mutagenicity) and cardiotoxicity (hERG-I & II inhibition) [11] were predicted. The properties were also predicted for standard drugs and used for comparison.

3 RESULT AND DISCUSSION

The preliminary phytochemical screening was carried out using the various extracted fractions. The phytochemical analysis gives the general idea regarding the nature of chemical constituents of the samples. The methanolic extract of C. asiatica leaves show the presence of glycosides, saponins, alkaloids flavonoids and steroids (Table -1)

TABLE 1
PHYTOCHEMICALS PRESENT IN THE CAMLE

SL NO	Phytochemicals	Test result
1	Phlobatannin	++
2	Glycoside	++
3	Saponin	++
4	Flavonoid	++
5	Alkaloid	++
6	steroids	++
7	Phenol	++

Protease inhibitory activity was performed using CAMLE

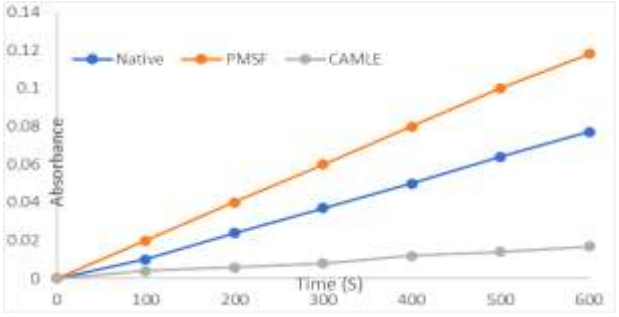

Fig. 1. Protease inhibitory activity of CAMLE and PMSF

and graph was plotted based on the values obtained at different time period (Fig 1). The percentage of inhibition was calculated from the formula and it was observed that the CAMLE showed 67.29% protease inhibition. On the other hand, the standard inhibitor (PMSF) exhibited only 53.2% inhibition.

Purification of compounds from Centella asiatica methanolic extract showing protease inhibitory activity was carried out by column chromatography using silica gel of different mesh sizes. The fractions were eluted using different solvent system based on the increasing polarity index (100% Hexane to 100% Chloroform) and was evaluated for protease inhibition using spectrophotometric time scan assay. Fractions showing the

Fig. 2. TLC image of the active fraction at 366 nm

similar banding pattern in TLC analysis were pooled together and subjected to preparative TLC. The compounds present in the active fraction obtained after preparative TLC were analysed by GC MS analysis.

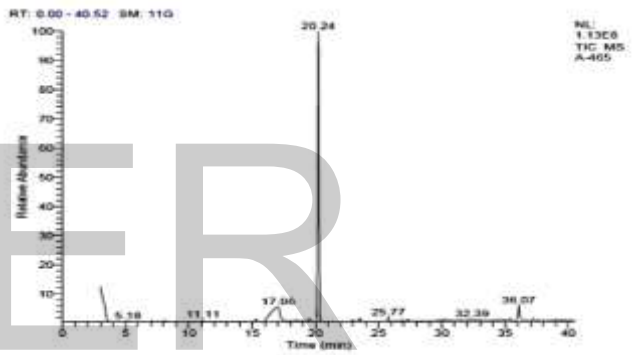

Fig. 3. a. GC MS chromatogram of active fraction of CAMLE

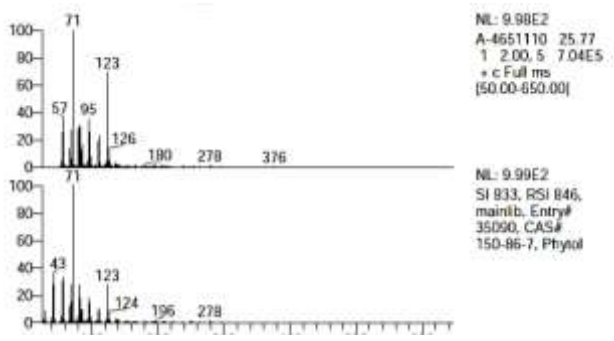

Fig. 3. b. MS analysis of the phytol

The GC MS analysis of the active fraction of Centella asiatica. indicated the presence of 7 major compounds of which phytol is the major constituent (Figure 3.a). Details of the MS analysis of phytol is given in figure 3.b.

The in-silico docking studies conducted using the identified compound (phytol) and the standard (PMSF) against Bovine trypsin (Fig. 5a – b) revealed that they interact with the same hydrophobic pocket that is involved in the catalytic activity of the protein. Even though both the compounds interact with the same

binding site, considerable differences were observed in the

binding free energy. The interaction of phytol seems thermodynamically favorable with more negative binding free energy (-5.351) than the standard (-4.1745) which is reflected in the negative glide score.

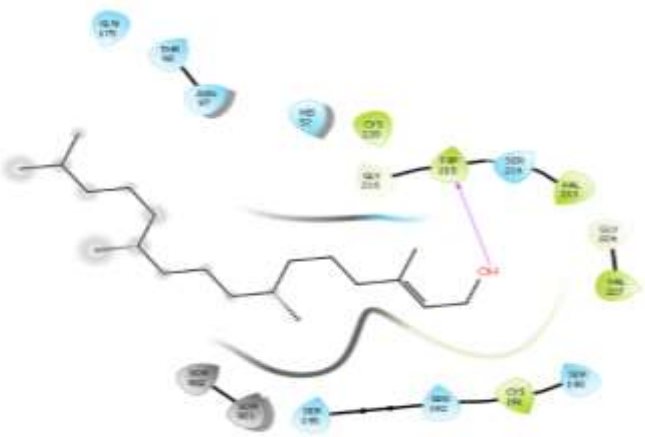

Fig. 4 a. Phytol trypsin interaction

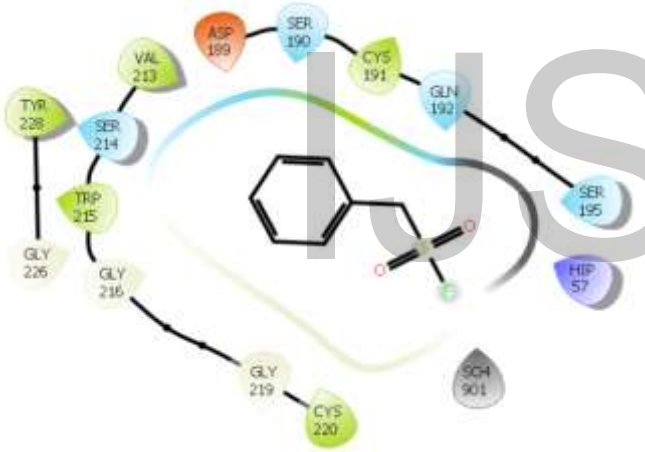

Fig. 4 b. PMSF trypsin interaction

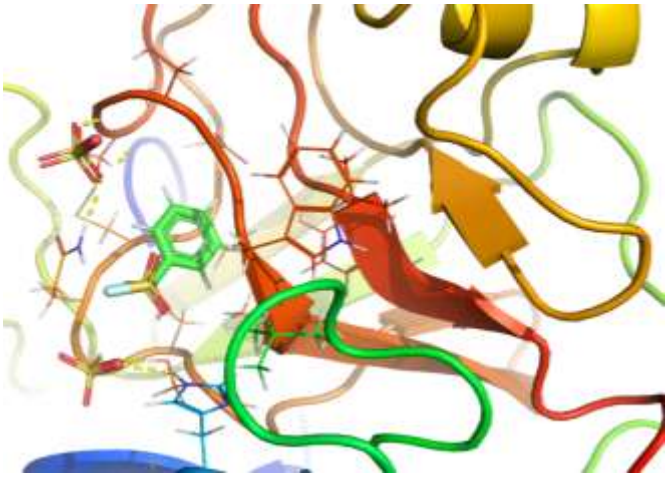

Fig. 5 a. Cartoon simulation of PMSF with bovine trypsin

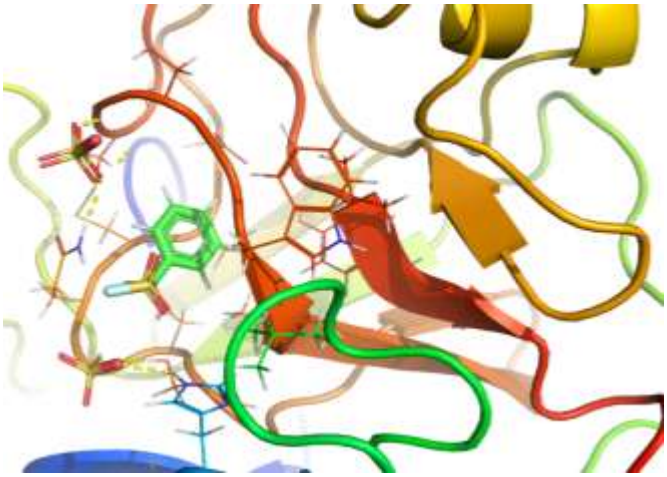

Fig. 5 b. Cartoon simulation of phytol with bovine trypsin

ADMET parameters of phytol were predicted using the swiss doc web server. ADME parameters such as CaCo-2 permeability, water solubility, intestinal absorption, P-glycoprotein, blood-brain barrier (BBB), the volume of distribution and CNS permeability along with Toxicity parameters such as and cardiotoxicity (hERG-I & II inhibition) AMES toxicity (mutagenicity). There is no toxicity was observed.

4 CONCLUSIONS

These experimental results partially validate the folklore claim of *Centella asiatica* leaves to be used as an anti-inflammatory agent. In vitro and in silico studies conducted provide a new scientific information about the anti-inflammatory activity of methanolic leaf extract of *Centella asiatica*. These activities can be attributed to the various phytochemical constituents present in the extract. Further detailed studies on the compounds present in the extract may eventually lead to the identification of novel compound/compounds helpful in the development of a drug against inflammation.

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